The immune system's contribution to the clinical efficacy of EGFR antagonist treatment

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Abstract

Epidermal Growth Factor Receptor (EGFR) antagonists were one of the first anti-cancer treatments developed targeting a Receptor Tyrosine Kinase. However, the underlying mode of action of how EGFR antagonist application can explain its clinical efficacy in different types of cancers remains largely unresolved. Numerous findings have suggested that a substantial portion of the effects attributed to EGFR antagonist treatment might not be based on *direct* influence on the tumour itself. Instead it may be based on *indirect* effects, potentially mediated via the immune system. In this review the role of the EGFR for the functioning of the immune system is discussed, alongside how EGFR antagonist treatment could be impacting tumour growth by blocking macrophage and FoxP3-expressing regulatory CD4+ T cell function. Based on these findings, we consider implications for current treatment schemes and suggest novel approaches to improve the efficacy of EGFR antagonist treatment in the future. Finally, we propose potential ways to improve EGFR antagonists, in order to enhance their clinical efficacy whilst diminishing unwanted side effects.

The Epidermal Growth Factor Receptor (EGFR) was the first Receptor Tyrosine Kinase to be described (1). Due to the fact that many tumours of epidermal origin express high levels of this cell surface receptor, antagonists targeting the EGFR were also amongst the first biologicals approved for the treatment of cancer patients (2). Examples of such antagonists include two EGFR blocking antibodies, Cetuximab and Panitumumab, as well as two chemical tyrosine kinase inhibitors, Erlotinib and Gefitinib. Many further antagonists are now in advanced development. These EGFR antagonists show considerable clinical efficacy and, in particular, their use in colon carcinoma as well as that in head and neck cancer treatment can substantially extend survival time (2).

Fundamentally, the clinical efficacy of EGFR antagonists in cancer treatment was an unexpected finding, as the EGFR is ubiquitously expressed throughout the human body and not itself an oncogene. Contrary, deletion of the EGFR specifically in hepatocytes has been shown to lead to enhanced development of liver cancer in mice (3), demonstrating that this receptor may have beneficial properties in the protection against cancer**.** Only a mutated form of the EGFR, known as EGFRvIII, can cause cancer. This mutation deletes exons 2 to 7 of the EGFR gene, leading to low-level constitutive signalling that can drive tumour progression (4).

A number of different direct modes of action have been suggested that may explain the clinical efficacy of EGFR antagonist treatment in cancer therapy. One of the original suggestions was that tumours grow “addicted” to growth factor signalling. As such, interruption of this signalling was assumed to lead to cancer cell death. However, this suggestion was substantially based around the fact that many tumours strongly overexpress the EGFR, and it was assumed that such an extreme overexpression of this receptor would supposedly lead to higher sensitivity of the tumour cells to growth factor-induced proliferation. Nevertheless, it was soon recognised that tumour-specific overexpression of the target molecule was not a prognostic marker of tumour treatment (5)(6), as tumours that did not express detectable levels of the EGFR still reacted to antagonist treatment such as monoclonal antibody therapy (7). It has also been shown that tumours responsive to EGFR antagonist treatment *in vivo* are often not sensitive to monoclonal antibody treatment in cell culture when explanted (8).

As alternative modes of action, in particular with antibody-based treatments, complement-mediated and natural killer (NK) cell-mediated killing of tumour cells has been suggested. However, as the EGFR is also ubiquitously expressed on healthy tissues, such modes of action would only be able to explain for the clinical efficacy of treatment on tumour cells that express an elevated level of the target molecule. Thus, it appears reasonable to assume that the clinical efficacy of EGFR antagonist treatment may not be based on *direct* effects on the tumour, but may also be in part based on *indirect* effects. One such possibility may be the interaction of antagonists with EGFR expression on healthy cells in the tumour microenvironment, such as tumour-intrinsic fibroblasts, or on cells of the immune system. In support of such an assumption it has been reported that

Cetuximab treatment in particular can activate the host anti-tumour immune response (8)(9). Furthermore, Garrido et al. demonstrated, in a mouse model of lung carcinoma, that the anti-metastatic effect of EGFR inhibitor treatment crucially depends upon the immune system (10). Depletion of either CD8+ or CD4+ T cells abrogated the beneficial effects seen following EGFR inhibitor treatment (10), therefore these findings strongly suggest that the immune system may substantially contribute to the clinical efficacy of EGFR antagonist treatment.

This review will discuss evidence that implicates the involvement of the immune system in EGFR antagonist-based tumour treatment, considering the measures required to improve current treatment in order to enhance clinical efficacy and diminish any associated side effects.

Role of the EGFR in the immune system

It has been well established that the EGFR is expressed on many different haematopoietic cell types and that its expression is of central importance for their functioning. These cell types include macrophages (11)(3), monocytes (12), plasma cells (13) and certain T cell subsets such as effector CD4 T cells and FoxP3-expressing regulatory CD4 T cells (Tregs) (14). It is therefore plausible that EGFR antagonists used to target tumours can interfere with the functioning of the immune system. This potentially explains for the enhanced susceptibility to infections seen in patients treated with these antagonists (15), and the observed mortality of patients arising from severe bacterial infections when treated with immunosuppressant mTOR inhibitors in combination with EGFR inhibitors (15).

Tumours require interaction with many different host immune cell populations for their growth and survival. Mast cells, for instance, are recruited to tumour environments where they mature and release angiogenic mediators to support the development of new blood vessels and provide growth factors to support tumour development (16). Tumour associated macrophages (TAM) are another key immune cell type implicated in tumour growth. TAMs have been found to stimulate angiogenesis, as well as secreting molecules that enhance tumour cell proliferation and metastasis, and promoting tumour progression by establishment of a suppressive microenvironment (17). Additionally, other suppressive cell types are also found in the tumour microenvironment, including regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). These cells dampen the anti-tumour immune response by interacting with cells such as NK cells, T cells and dendritic cells (DC) (18)(19). Thus, it is reasonable to assume that EGFR antagonist interference with any of these leukocytes' function may advantageously contribute to the clinical efficacy of anti-tumour treatments.

Role of EGFR expression for macrophage function

Macrophages make up a substantial component of many tumours. Clinical studies have indicated that cancers containing high infiltrates of macrophages are associated with poor prognosis for the

patient (20). Recent studies in a number of different mouse tumour models have revealed that EGFR-mediated signalling, specifically within macrophages, substantially contributes to the initiation of tumour growth (3)(21)(22). In line with these findings, the group of John Condeelis revealed an EGFR based cross-talk between macrophages and tumour cells (23). In a paracrine loop intra-tumoural macrophages secrete EGF, which binds to the EGFR on tumour cells, promoting their invasion. At the same time, tumour cells secrete colony-stimulating factor-1 (CSF-1) which in turn promote the expression of EGF by macrophages (23). Nevertheless, in all of the mouse tumour models used, EGFR gene-deletion in the cancerous target tissue had no influence on the development of tumours. However, EGFR gene-deletion in macrophages did influence tumour development. This suggests a role for macrophage-intrinsic EGFR-mediated signalling in the establishment of the tumour microenvironment. Mechanistically it has been shown that EGFR expression regulates macrophage cytokine production (3)(24), and that macrophage-derived cytokines are predominant drivers of tumourigenesis (3)(21)(22). Thus, by blocking EGFR function in tumour residential macrophages during EGFR antagonist treatment this supply of cytokines may be interrupted, leading to diminished tumour growth and tumour-intrinsic instability.

Role of EGFR expression for regulatory T cell function

One further immune cell population of particular interest in cancer treatment are FoxP3-expressing regulatory T cells (Tregs). The role of EGFR expression for Treg functioning is also well established (14) and Tregs play a role in maintaining immune homeostasis by negatively regulating other immune cell types. Thereby Tregs prevent the development of autoimmunity through establishment of peripheral tolerance to self-antigens. Tumours are able to exploit the suppressive nature of Tregs found within the tumour microenvironment, and Tregs have been shown to traffic to, proliferate and mature within the tumour microenvironment under influence of local factors produced by tumours and associated cells (18). As such, the presence of Tregs allows tumours to escape immune surveillance as they dampen anti-tumour immune responses, enabling the induction of immunological tolerance against tumour-antigens.

For optimal functioning, both human and murine Tregs are dependent on EGFR-mediated intrinsic signalling upon binding of the ligand Amphiregulin (AREG)(14). AREG is known to be a type II cytokine involved in wound healing and contributes to host resistance to helminth infections (25)(26). In contrast to most other EGFR ligands, the binding of AREG to the EGFR induces a prolonged, tonic signal through the MAP-kinase (MAPK) signalling pathway that does not lead to the internalisation of the receptor. Tregs express the EGFR under inflammatory conditions, as witnessed by elevated levels of EGFR found on tumour-infiltrating Tregs derived from wild type (wt) mice with B16 melanomas, as well as EGFR expression on human Tregs with an activated phenotype of FoxP3^{hi} and CD45RA- (14). *In vitro* treatment of EGFR-expressing Tregs with AREG

enhances their suppressive capacity, shown by decreased proliferation of peripheral blood mononuclear cells (PBMC) incubated with both Tregs and AREG. The group of Mark Wilson further demonstrated that the addition of AREG increases the release rate of microRNA-containing exosomes from Tregs, which these cells use as a means of Treg-mediated immune suppression (27). In consequence, Tregs that either lacked EGFR expression or were transferred into an AREG-deficient environment could not suppress the development of autoimmune diseases in both a dermatitis and a T-cell transfer-based model of colitis (14). This clearly demonstrates that Tregs are directly dependent on AREG-induced and EGFR-mediated signalling for their *in vivo* functioning.

It is widely accepted that Tregs play a pivotal role in creating the suppressive tumour microenvironment. Using a well-established murine B16 melanoma immunisation model (28), it has been demonstrated that AREG plays a critical role in such Treg-mediated tumour immune suppression (14). In B16 tumours, Tregs protect against immunisation-induced CD8+ T cell-mediated anti-tumour immune responses. It has been shown that when C57BL/6 wt mice were transplanted with B16 tumours, and an anti-tumour CD8+ T cell response induced by immunisation with immunogenic tumour-epitope pulsed bone marrow-derived dendritic cells (BM-DC), large tumours grew 3 weeks following tumour transfer. In contrast, BM-DC immunised *Areg* gene-deficient mice and mice lacking EGFR expression specifically on Tregs (*FoxP3-cre x* $EGFR^{fl/fl}$)(unpublished data) efficiently rejected the transplanted tumours upon immunisation (14). These findings demonstrate that AREG enables Tregs to suppress anti-tumour immune responses.

Remarkably, mast cells have been determined to be the critical source of AREG, enhancing Treg function (14). It has been shown before that mast cells can cooperate with Tregs to suppress skin transplant-specific immune responses, a model in which the immunosuppressive function of Tregs is well established to induce immunological tolerance (29). Furthermore, in a model of T cell transfer-induced colitis, using a mast cell-deficient mouse strain *c-kit*$^{w\text{-}sh/w\text{-}sh}$ backcrossed onto a *RAG1*$^{-/-}$ background, it was demonstrated that mice reconstituted with *wt* bone marrow-derived mast cells (BM-MC) prior to co-transfer of naïve CD4+ T cells and Tregs had a much lower colitis score than mast cell-deficient mice that had either not been reconstituted or reconstituted with *Areg*$^{-/-}$ BM-MC. This clearly demonstrates that mast cell-derived AREG is essential to restore the suppressive capacity of transferred Tregs (14).

In further accordance with these findings, it has also been demonstrated that the efficacy of tumour immune therapy is enhanced in mast cell deficient mouse strains (30)(14). Mast cells are found to accumulate at the edge of tumours, and in some tumour types this accumulation has been shown to correlate with a poor prognosis for cancer patients (31). Tumour-associated Tregs have also

been shown to localise on tumour margins, within so-called tertiary lymphoid structures (TLS)(32). TLSs are often formed at sites of infection, inflammation and cancer, and similarly to secondary lymphoid structures they contain B cell and T cell zones along with high endothelial venules. This composition allows for the formation of efficient adaptive immune responses, as TLSs allow entry of immune cells into the tumour environment and priming of lymphocytes. It is here that Tregs are believed to suppress anti-tumour immune responses. Reconstitution of mast cell-deficient *c-kit*$^{w\text{-}sh/w\text{-}sh}$ mice with *wt* BM-MC prior to tumour transfer, and immunisation with tumour-epitope pulsed BM-DCs, led to enhanced resistance to tumour immune therapy. However, when *c-kit*$^{w\text{-}sh/w\text{-}sh}$ mice were reconstituted with *Areg*$^{-/-}$ BM-MC prior to immunisation, tumour protection against the induced immune responses was lost. These findings highlight that mast cell-derived AREG increases the suppression of Tregs *in vivo,* thereby enabling tumour-resident Tregs to suppress anti-tumour immune responses (Figure 1).

Taken together, targeted interference with Treg-intrinsic EGFR signalling in human tumours may as such contribute to the observed efficacy of EGFR antagonists. Consistent with such an assumption is the fact that the best independent prognostic indicator for the efficacy of treatment in patients with tumours expressing a non-mutated form of K-Ras, is the expression level of AREG in the serum of cancer patients (33)(34)(35)(36).

Role of regulatory T cells in human cancer

In mouse tumour models the importance of Tregs has repeatedly been demonstrated. In contrast, their importance in established human tumours remains a more controversial and less well studied subject. An elevated number of FoxP3-expressing CD4+ T cells have been described within several solid tumours; including ovarian and non-small-cell lung cancer tumours, and in the peripheral blood of breast, colorectal, and lung cancer patients (18). Data from patients suffering from a wide range of cancer types has further indicated that there appears to be a positive correlation between increased number of intra-tumoural FoxP3-expressing CD4+ T cells and poor prognosis for cancer patients (37). Unfortunately, both Tregs and activated human CD4+ T cells transiently express the transcription factor FoxP3 (38). Therefore, an exact determination of the T cell phenotype of human FoxP3-expressing CD4+ T cells requires the analysis of the methylation status of the FoxP3 gene locus (39). Such analysis relies upon an assay only rarely performed on patient biopsy-derived T cells. In addition, the relevance of Tregs may be less important in the situation of established tumours that have already managed to circumvent and tolerise potential immune responses. Together this suggests that while Treg function may play a critical role at specific time points during the establishment of tumours, at the exact point at which the biopsy are taken Treg function may no longer play an essential role for tumour growth.

Nevertheless, clear evidence for the importance of Treg function during tumour immunotherapy exists. In recent years, the use of anti-CTLA-4 antibodies has gained significant support and success in the treatment of several types of tumours; supposedly, by reinvigorating tumour-specific cytotoxic CD8+ T cells suppressed by CTLA-4-expressing Tregs (40). Furthermore, the importance of Tregs in limiting the efficacy of immunotherapy has been well demonstrated in clinical cancer therapy. For example, it has been reported that the efficacy of vaccination against a high risk HPV type (HPV16) in patients with cervical cancer was directly correlated with the frequency of FoxP3-expressing CD25+ CD4+ T cells in patient peripheral blood following vaccination (41). Also, in clinical studies in which patients received chemotherapy followed by adoptive immunotherapy, the level of Treg reconstitution after transfer appeared inversely correlated with patient response to therapy (42). Thus, it is now assumed that the balance between effector T cells and Tregs may determine the impact of the anti-tumour therapy.

Collectively, these findings strongly suggest that Treg function might also play an important role in human tumour immune therapy, and we may assume that EGFR antagonist treatment-associated interference with Treg-mediated immune suppression enhances the efficacy of such cancer treatments.

Improved applications of EGFR antagonists

The knowledge that EGFR inhibitor treatment may suppress the function of Tregs, thus aiding in improving the efficacy of tumour immunotherapy, allows the novel exploration of combination therapy. By combining current therapies with the use of EGFR antagonists, enhanced anti-tumour immune responses in cancer patients may be seen (Figure 2).

One such therapy includes tumour vaccination, which in the past has often failed in clinical applications, potentially due to a Treg-mediated immunosuppressive tumour microenvironment. It has already been demonstrated in a mouse model of B16 melanoma that combined therapy of tumour immunisation with EGFR antagonist treatment improves the efficacy of either treatment alone (14). B16 tumours were transferred into *wt* C57BL/6 mice, and the mice later immunised with either tumour antigen-pulsed BM-DCs or EGFR-blocking nanobodies, BM-DCs in combination with EGFR-blocking nanobodies, or alone. Mice that were given nanobodies or antigen-pulsed BM-DCs alone showed little to no regression of tumour growth. However, mice that were immunised with BM-DCs in combination with nanobody treatment developed significantly smaller tumours; demonstrating the improved efficacy of combined therapies in this mouse model (14).

Another such treatment that could be combined with current EGFR inhibitor therapy is chemotherapy (CT). CT is currently one of the most common forms of cancer treatment, with the efficacy of CT depending on the type of cancer and the stage of tumour development. CT has a variety of effects on the immune system including, of importance, immunosuppression through

depletion of immune cells such as dividing T cells. The group of Zitvogel and Kroemer have shown elegantly that CT can also induce and enhance anti-tumour immune responses in a number of ways (43). Firstly, CT induces tumour cell death. As such, tumour (neo-) antigens are released and can subsequently be presented to tumour-specific T cells priming new immune responses against tumour cells or re-activating a dormant anti-tumour T cell response. In addition, CT-induced tumour cell death diminishes the overall tumour load temporarily, and thus with it the associated immunosuppressive microenvironment. However, most importantly for combined immunotherapy, CT transiently induces lymphopenia. This leads to substantial T cell proliferation that can again reactivate dormant T cell responses. It has further been well established that in situations of lymphopenia, the activation of Treg populations plays an important role in dampening such dormant responses; for instance, to prevent the induction of autoimmune responses (44). The application of EGFR antagonists, and thus the suppression of Tregs function during this immune replenishment phase following CT-induced lymphopenia, may have the capacity to substantially improve the reactivation of anti-tumour immune responses and efficacy of CT (Figure 2). Of interest, in colon carcinoma patients, EGFR antagonist treatment appears efficient only in combination with chemotherapy.

Another promising line of combined therapy in cancer treatment is the combination of CT with adoptive cell transfer (ACT). In most cases, ACT describes the isolation of T cells from cancer patients. These T cells are then further cultured *in vitro,* and subsequently activated and expanded *ex vivo* with tumour-specific antigens prior to re-infusion. Alternatively, effector T cells are transduced with tumour-antigen specific TCRs eliciting a stronger anti-tumour immune response following later retransfer into the patient (45). ACT is normally combined with non-myeloid depleting CT prior to cell transfer, inducing lymphopenia-associated cell proliferation. One of the most critical aspects in determining the efficacy of ACT is thereby the rate in which Tregs expand following CT (42). Patients with a rapid recovery of Treg frequencies in the blood following therapy were mainly non-responders, while patients who had a delayed Treg recovery were responders (42). Therefore, it is tempting to speculate that the application of EGFR antagonists in combination with ACT during CT-induced lymphopenia, and the following recovery phase, could substantially diminish Treg function during a critical time point of treatment thus substantially improving the efficacy of such treatment (Figure 2).

Outlook

Taken together, recent developments suggest a prominent immune involvement in the clinical efficacy of EGFR antagonist treatments. To fully verify such an involvement, it appears warranted that further and more focused analysis of tumour material - derived from cancer patients treated with EGFR antagonists - should be performed. These studies should address EGFR expression levels of intra-tumoural Treg populations during different stages of tumour development, and

determine whether a shift in effector T cell to Treg ratio occurs in patients that undergo treatment with EGFR antagonists. Nevertheless, this newfound appreciation of the link between EGFR inhibitors and the immune system provides novel insights into how EGFR antagonists may function during cancer treatment, permiting adjustments to our current use of these drugs in the clinic. As the EGFR is expressed on a wide variety of cells, and is important for tissue homeostasis, off-target side effects of current EGFR inhibitors used in cancer treatment are common and can often be severe. To diminish such side effects, methods are required to be developed which can target EGFR antagonists specifically to tumour-residential Treg populations. As such, much lower concentrations of drugs would be needed as their effects would remain restricted to Tregs; thus enhancing the efficacy of treatment whilst diminishing side effects associated with EGFR antagonist treatment.

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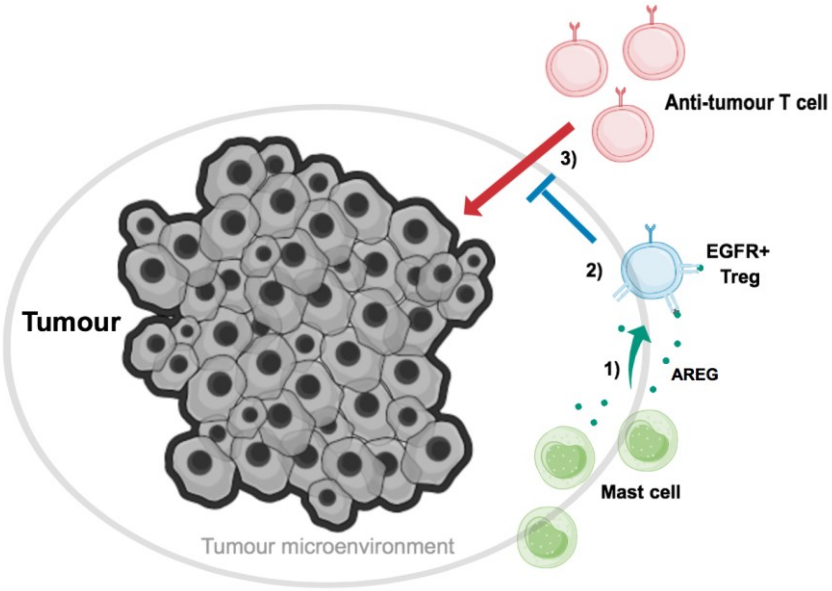

Figure 1: Mast cell-derived Amphiregulin enables tumour-residential regulatory T cells to suppress anti-tumour immune responses

Tumour-associated Tregs (blue) have been found to localise on tumour margins, along with mast cells (green). **1)** Mast cells cooperate with Tregs, and aid in tumour growth, through secretion of the EGFR-ligand AREG (dark green). **2)** AREG binds to EGFR on Tregs, inducing intrinsic signalling which ultimately enhances Treg suppressive function. **3)** Tregs, with enhanced EGFR-mediated suppressive abilities, can now suppress the action of anti-tumour T cells, and thus aid in tumour immune evasion.

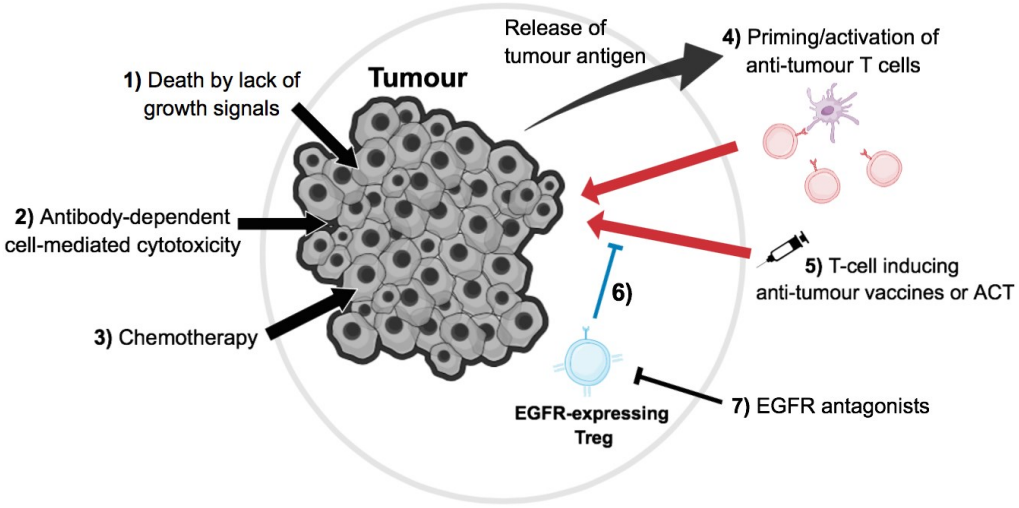

Figure 2: Direct and indirect effects of tumour immunotherapy

1) For a subset of EGFR+ tumours, growth will be dependent on signals transmitted via the EGFR; therefore blocking this signal with EGFR antagonists could induce tumour cell death by lack of growth signals. **2)** EGFR antagonists, such as monoclonal antibodies, can activate natural killer cell-mediated antibody-dependent cell death (ADCC) on EGFR-expressing cells, such as tumour cells and other immune cell populations in the microenvironment, thus ultimately leading to tumour cell death. **3)** Chemotherapy is a common form of cancer treatment, which has a variety of effects such as tumour cell death and lymphopenia, which re-invigorates the anti-tumour T cell response. **4)** Tumour cell death leads to the release of antigens, which can be presented by antigen presenting cells such as dendritic cells (purple) to prime or re-activate anti-tumour T cells (red). **5)** Anti-tumour T cells can also be indirectly induced or directly transfused via vaccines or adoptive cell therapy respectively, which then can kill tumour cells. **6)** These immune responses can be blocked by tumour-residential regulatory T cells (blue); however, **7)** EGFR antagonists can hinder the suppression of Tregs that is induced via EGFR signalling, thus in turn aiding in tumour immunotherapy, as these Tregs no longer suppress anti-tumour T cells as efficiently.